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# Determining the Effects of Quercetin on Cadmium Toxicity in Kidney Cells

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**ABSTRACT**

Cadmium is a heavy transition metal that causes kidney disease via prolonged, low-level exposure due to circulating metallothioneins. These proteins transport cadmium ions to the proximal convoluted tubule, where they induce the creation of reactive oxygen species (ROS). Oxidative damage from ROS can lead to kidney dysfunction and eventually failure. Previous studies have shown that antioxidants, including quercetin, which is found in most fruits and vegetables, can lessen cadmium-induced toxicity. In this study, human embryonic kidney cells were pretreated for one hour with varying concentrations of quercetin ranging from 10 - 100 μM. This was followed by 24-hour treatment with 30 μM cadmium chloride, and growth was measured by a cell proliferation assay. In contrast to previous reports, these studies suggest that quercetin concentrations above 10 μM result in an increase in cell death, suggesting that quercetin enhances, rather than inhibits, the toxic effect of cadmium within this concentration range. To determine if cadmium toxicity induced cell death through activation of the JNK pathway, the level of JNK phosphorylation was measured by immunoblot analysis. These results suggest that cadmium alone increases JNK phosphorylation slightly while quercetin activates JNK in a concentration-dependent manner. This activation was increased up to thirteenfold when treated with both cadmium and quercetin. Taken together, these studies indicate that cadmium toxicity induces cell death via the JNK pathway, that quercetin above 10 μM also exhibits cytotoxic effects through JNK phosphorylation, and that quercetin and cadmium act synergistically to lower cell proliferation.

# INTRODUCTION

## Heavy Metals and the Risks Posed

Heavy metals are a group of toxic metals, including cadmium, arsenic, lead, beryllium, and mercury, that can harm human health. In small quantities, some of these metals are important components in biological molecules such as enzymes that are necessary for life and different cellular processes. According to the Occupational Safety and Health Administration (OSHA), however, large amounts can become hazardous to organisms if accumulation is allowed to occur in their organ systems (2018). Common patterns of exposure to heavy metals include inhaling airborne metals, ingesting contaminated food or water, and chronic exposure to low levels (Prozialeck *et al.*, 2006). The first two of these patterns make all animals and plants susceptible to the hazards posed by increased concentrations of heavy metals, but chronic exposure to low levels is unique to humans, particularly to those who work in industrial fields.

As an environmental health problem, heavy metal pollution is largely due to the smelting and electroplating industries. All of the following areas contribute to this issue: the manufacture of batteries, dyes, paints, and plastics; the burning of heavy metal-containing waste; the use of contaminated sludge and phosphate salts as fertilizers; and tobacco use (Prozialeck *et al.,* 2006; Wang *et al.*, 2017). One of the primary metals involved in these methods of industrial pollution is cadmium.

## Cadmium

Cadmium is a Group 12 heavy transition metal that often exists as a cation with a 2+

charge, and it does not naturally occur in biological systems, according to the National Center for Biotechnology Information (NCBI) (2018). The industrial processes unique to cadmium pollution include refining metals and burning fossil fuels (Wang *et al.*, 2017). These industrial methods release cadmium particles into the environment, allowing cadmium to permeate the soil (Hashemi and Farajpour, 2016). From that point, there is a high rate of soil-to-plant transfer, which results in contamination of the surrounding plants and agriculture (Wang *et al.*, 2017). If contaminated produce reaches grocery store shelves, the accompanying cadmium can enter human organ systems. Lastly, cadmium is classified by the International Agency for Research on Cancer as a group I carcinogen, meaning it conclusively causes cancer in human beings (Wei-ping *et al.*, 2007).

Cadmium *in vivo* primarily causes disease via prolonged, low-level exposure (Prozialeck *et al.,* 2006; Wang *et al.*, 2017; Fujiwara *et al.*, 2012). This is possible due to a family of proteins called metallothioneins. Metallothioneins are found in several different species of animals, and they are used for transporting very small amounts of heavy metals in the body that are necessary for cellular processes (Davis and Cousins, 2000). These proteins are rich in cysteine residues, which give them the ability to chelate heavy metal ions, as seen in Figure 1 (Grennan, 2011; Stillman, 1995). Cadmium is one of these heavy metal ions, and binding to metallothionein gives it the ability to move through systemic circulation (Prozialeck *et al.*, 2006; Renugadevi and Prabu, 2009). More specifically, cadmium binds to metallothionein in the gastrointestinal tract,

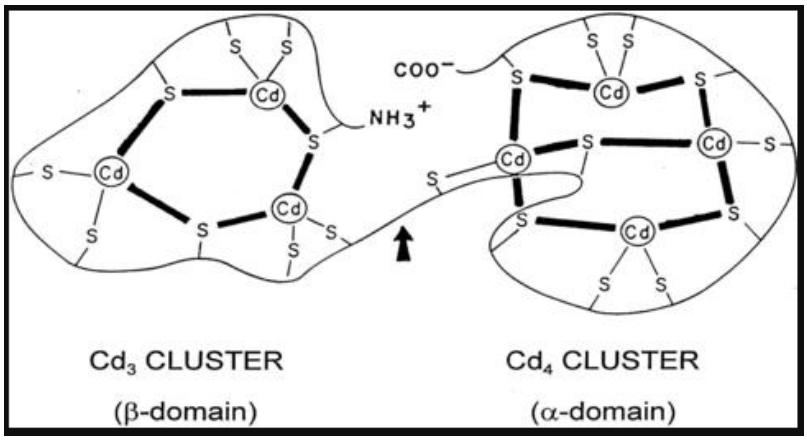


**Fig. 1. General binding structure of metallothionein to cadmium.** (Stillman, 1995)

and the cadmium-metallothionein complex moves through the liver and heart to deposit itself into the kidneys (NCBI, 2018). When present in the body, cadmium's biological half-life extends from ten to thirty years (Prozialeck *et al.*, 2006; Wang *et al.*, 2017; Fujiwara *et al.*, 2012). During this extended period of time, cadmium is able to cause disease by inducing oxidative stress (Fujiwara *et al.*, 2012; Prabu *et al.*, 2013).

**Oxidative Stress**

Oxidative stress is defined as the disturbance in the balance between reactive oxygen species production and the cell's mechanisms for detoxification of those free radicals (Nelson and Cox, 2013). It is typically caused by continued oxidative phosphorylation in the mitochondria when there are higher concentrations of reduced ubiquinone than necessary to meet the energy needs of the cell (Nelson and Cox, 2013). The production of reactive oxygen species in oxidative phosphorylation is a nonenzymatic process of generating free radicals (Pham-Huy *et al.*, 2008). Enzymatic generation of free radicals, on the other hand, occurs in phagocytosis and prostaglandin synthesis to kill pathogens (Pham-Huy *et al.*, 2008; Robinson, 2008; Zhao *et al.*, 2013). Reactive oxygen species have also been implicated in aging due to the mitochondrial

dysfunction observed in diseases related to aging (Cui *et al.*, 2012).

Additionally, oxidative stress can be caused by heavy metal toxicity, particularly in organ systems that are regularly exposed to environmental pollutants (Dua *et al.*, 2015). Reactive oxygen species are produced when molecular oxygen is reduced to superoxide, often in the mitochondria (Figure 2) (Dua *et al.*, 2015; Dröge, 2002). Superoxide is the precursor of many reactive oxygen species, including hydrogen peroxide, and if left alone, these reactive oxygen species can cause oxidative damage (Nelson and Cox, 2013). In the case of cadmium toxicity, reactive oxygen species directly oxidize several biological macromolecules, particularly lipids, proteins, and nucleic acids (Donpugha *et al*., 2011). Destruction of these macromolecules

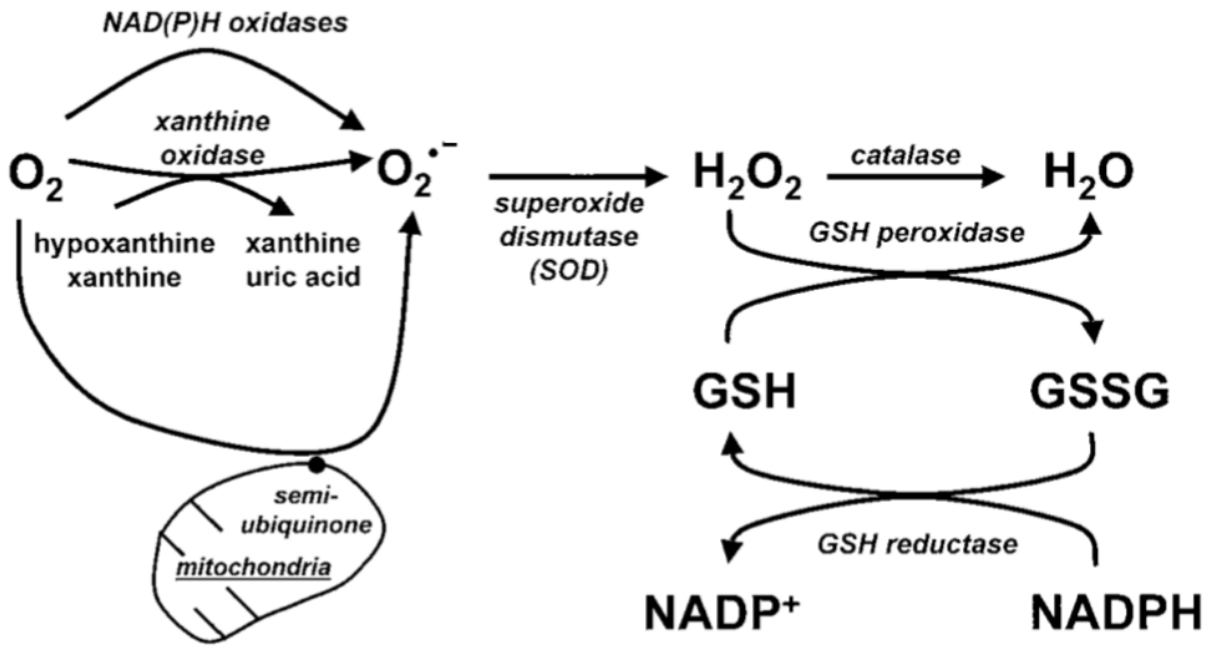


**Fig. 2. Pathways of reactive oxygen species production and clearance.** (Dröge, 2002)

damages larger internal cell structures, such as mitochondrial membranes and nuclear envelopes (Donpugha *et al*., 2011). In normal cases, the cell has built-in mechanisms part of the antioxidant

system to protect itself from oxidative damage (Li *et al.*, 2017; Dröge, 2002). In the presence of cadmium, however, production of reactive oxygen species has been shown to double (Dua *et al.*, 2015). These mechanisms are inadequate in protecting the cell's mitochondria in such extreme cases of oxidative stress, and the resulting mitochondrial dysfunction is a key contributor to programmed cell death, also called apoptosis (Prozialeck *et al.*, 2006; Dua *et al.*, 2015).

Apoptosis is one of two types of cell death (Nelson and Cox, 2013). This is a controlled process regulated by internal cell signaling, and it is an important aspect of normal cell turnover, growth, and development (Elmore, 2007). The first parameter for cytotoxicity that appears is typically cell shrinkage, which occurs before other characteristics of apoptotic cells (Nishimura *et al.*, 2008). These other characteristics are cytochrome c movement from the mitochondria, caspase activation, and DNA fragmentation (Nishimura *et al.*, 2008). The second type of cell death is necrosis, which is caused by external cellular injury (Grynberg *et al.*, 2017). While necrosis is considered the more toxic of these cell death mechanisms, there is overlap in the cell signaling used in both of these processes (Elmore, 2007).

**c-Jun N-terminus Kinase Pathway**

Mitochondrial dysfunction is linked to the JNK pathway, or the c-Jun N-terminus kinase pathway. The JNK pathway is a member of the mitogen activated protein kinase (MAPK) family, also known as the stress-activated protein kinase family (Chen *et al.*, 2016). Like other MAPK proteins, JNK plays an important role in the regulation of several cellular processes, including signal transduction, growth, and survival (Liu *et al.*, 2015). At the center of this

pathway is the JNK protein itself, which influences different cellular outcomes through its phosphorylation. Phosphorylation occurs at two different sites on the protein, Ser63 and Ser73 (Behrens *et al.*, 1999; Kanda and Miura, 2004). Once JNK is activated via phosphorylation, one possible result of this pathway is apoptosis, as seen in Figure 3 (Cell Signaling Technology, 2012). The JNK pathway can be activated by growth factors, ultraviolet and gamma radiation, inflammatory cytokines, and oxidative stress signals, including hydrogen peroxide and other reactive oxygen species. Reactive oxygen species first activate ASK1, which modifies the MAPK protein MKK4/7. MKK4/7 phosphorylates JNK at the two specific serine residues. From this point, phosphorylated JNK (P-JNK) can induce apoptosis in two ways. The first way is through the nucleus. P-JNK interacts with several transcription factors such as c-Jun and p53 to produce several pro-apoptotic factors, and these pro-apoptotic factors induce cell death. The second way P-JNK can cause cell death is through the mitochondria (Liu *et al.*, 2015). P-JNK interacts with the mitochondrial membrane proteins Bax, Bcl-2, Bim, and Bad, all of which normally help stabilize different regions of the mitochondrial membrane (Creative Diagnostics, 2010; Srivastava *et al.*, 1999). The role of Bcl-2, for example, is to stabilize cytochrome c (Srivastava *et al.*, 1999). Cytochrome c is vital to the cell's energy production because it transfers electrons from Complex III to Complex IV in the electron transport chain (Nelson and Cox, 2013). When JNK is activated by phosphorylation, it disrupts these proteins so that they cannot keep the mitochondrial membrane stable (Srivastava *et al.*, 1999). In the case of Bcl-2, JNK phosphorylation has been linked to the dislocation of cytochrome c from the mitochondrial

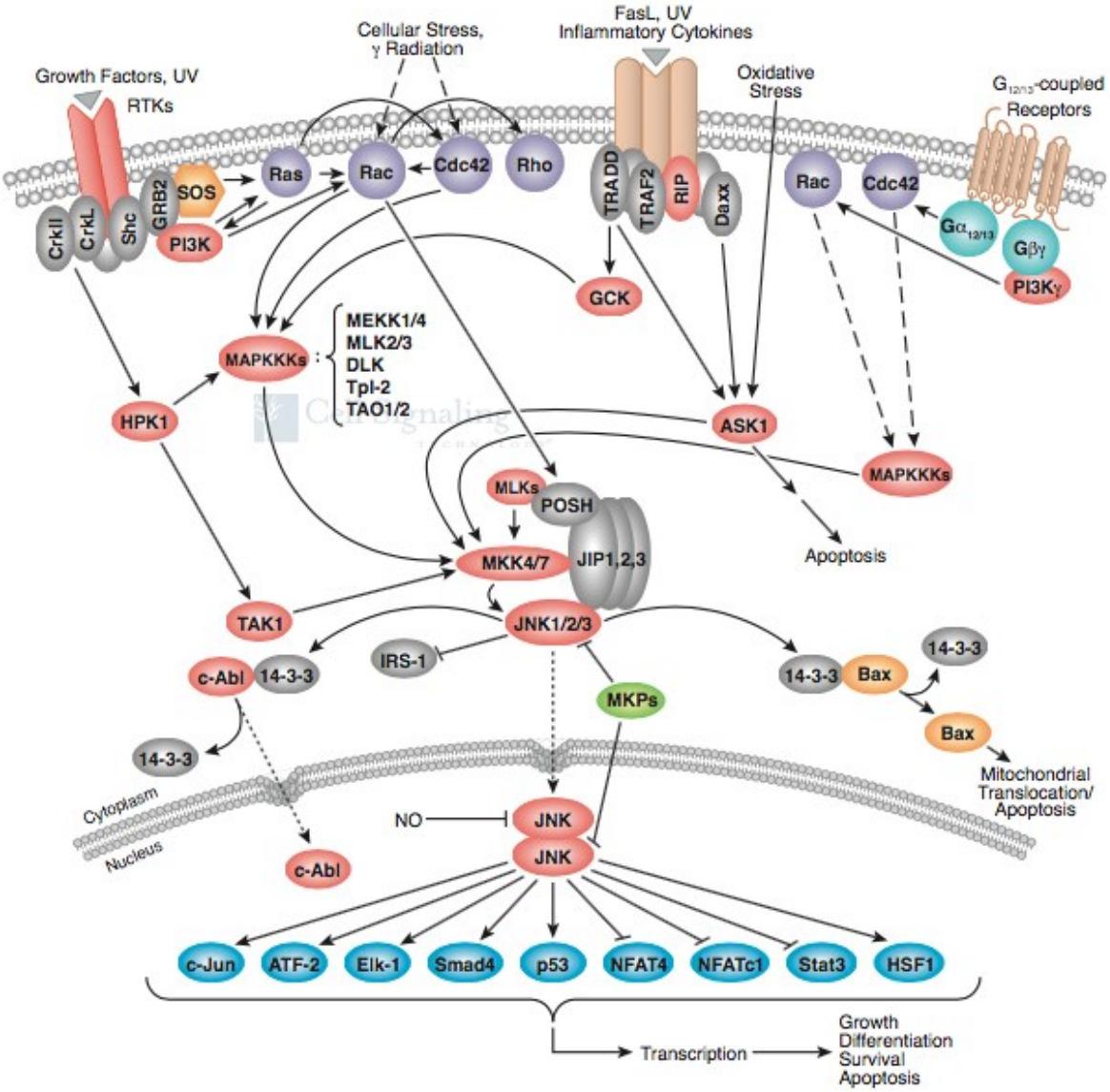


**Fig. 3. JNK signaling pathway.** (Cell Signaling Technology, 2012)

membrane to the cytosol (Jin *et al.*, 2006). Cytochrome c's dislocation is due to the malfunction of Bcl-2, and Bcl-2 is known to be inactivated by P-JNK (Xing-Ding *et al.*, 2009; Srivastava *et al.*, 1999). The movement of cytochrome c signals that the electron transport chain is unable to produce ATP. Without an appropriate amount of ATP, the cell is unable to continue the basic processes necessary for survival, and thus the cell undergoes apoptosis in order to protect the

surrounding tissue. JNK phosphorylation has also been implicated in necrosis; however, the dislocation of cytochrome c has been specifically linked to apoptosis (Grynberg *et al.*, 2017). Additionally, it has been shown that JNK phosphorylation increases in the presence of cadmium (Wang *et al.*, 2017; Chen *et al.*, 2016). As the concentration of cadmium increases, it is likely that the ratio of P-JNK to total JNK increases, and therefore cell death increases.

**Kidney Anatomy, Physiology, Pathology, and Cadmium Toxicity**

The outcomes of cadmium toxicity and the involvement of the JNK pathway are relevant because they affect the structure and function of the kidneys *in vivo*. The kidneys have two functions: to filter blood for the regulation of water, salt, acidity, and nutrients, and to excrete metabolic waste products and foreign chemicals (Pearce, 2008; Widmaier *et al.*, 2014). Concerning the gross anatomy, kidneys have an outer renal cortex and an inner renal medulla (Gosling *et al.*, 2017). Each medullary pyramid has a renal papilla at its tip. The papilla drains into minor and major calyces, which collect urine at the renal pelvis. The renal pelvis funnels into the ureter to transport urine to the bladder.

On a microscopic scale, kidneys are composed of functional units called nephrons (Figure 4). There are approximately 1.2 million nephrons in each kidney on average, and each nephron consists of a renal corpuscle and a tubule (Pearce, 2008; Widmaier *et al.*, 2014). The renal corpuscle, also called the glomerulus, filters the blood from the afferent arteriole and forms a filtrate of nutrients and waste. The tubule is divided into four parts: the proximal convoluted tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct. The proximal

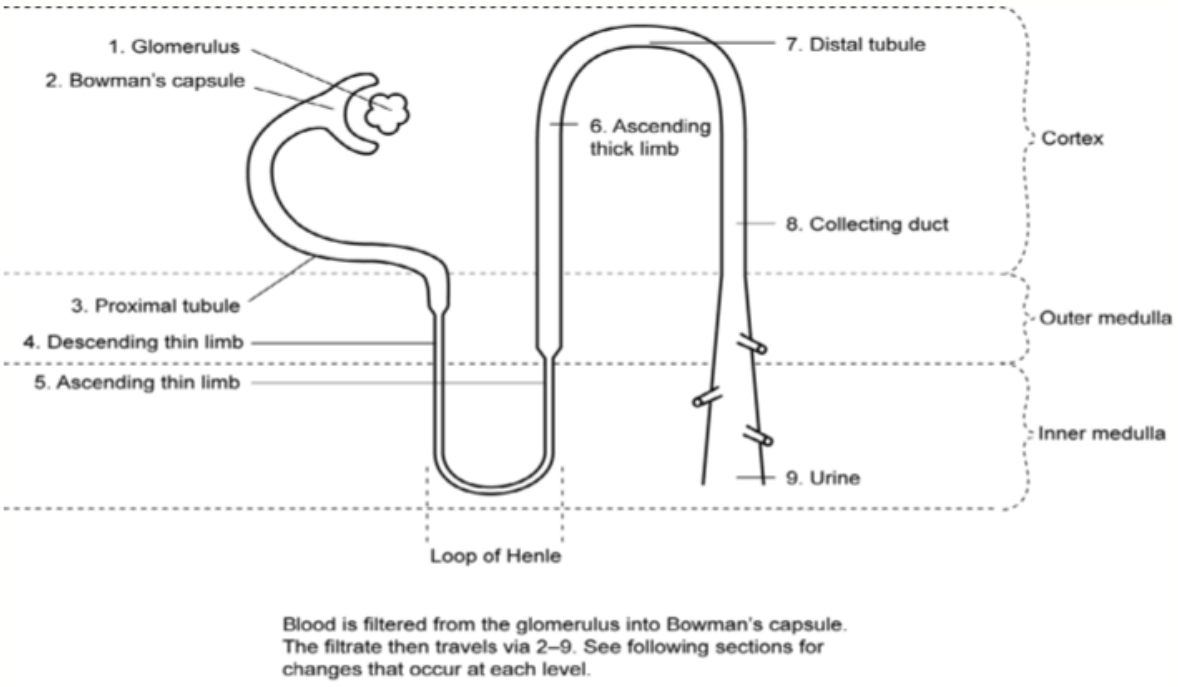


**Fig. 4. Constituents of the nephron.** (Pearce, 2008)

convoluted tubule and the distal convoluted tubule are located generally within the renal cortex, and the loop of Henle and the collecting duct can be found in the renal medulla. Each part of the tubule has a single layer of epithelium, which allows for an exchange of nutrients and waste between the filtrate and the blood in the peritubular capillary network. This epithelial cell layer, while small, is responsible for proper renal function, and injury to this tissue can result in kidney failure (Widmaier *et al.*, 2014).

There are two major types of renal failure: acute renal failure and chronic renal disease (Mayo Clinic, 2018 and 2015). Acute renal failure is the rapid onset of elevated blood urea nitrogen or creatinine in combination with a small amount or no urine production (Kemp *et al.*, 2007). This kind of kidney failure often occurs when a patient has presented to the hospital for a different medical condition typically requiring intensive care (Mayo Clinic, 2015). Conditions

like blood loss, myocardial infarctions, or glomerulonephritis can be precursors to acute kidney failure (Mayo Clinic, 2015). Treatment for acute failure is dependent on its cause, such as intravenous fluid to restore blood loss or medications to control electrolytes in the case of heart disease (Pearce, 2008).

While acute renal failure has a rapid onset, chronic renal failure consists of the slow development of renal fibrosis, which is scar tissue resulting from repeated inflammatory responses (Matovinović, 2009). The severity of chronic renal failure is classified by the glomerular filtration rate, or the rate at which the renal corpuscle filters the blood (Matovinović, 2009; Widmaier, 2014). A normal, healthy kidney has a glomerular filtration rate of greater than 90 mL/min., while in end-stage renal disease, the worst type of chronic kidney failure, the kidney has a glomerular filtration rate of 15 mL/min. or less. For end-stage renal disease, patients have only two options (Mayo Clinic, 2018). The first is a kidney transplant from a donor with a healthy kidney (Mayo Clinic, 2018). This procedure can be successful when medications are taken for the rest of the patient's life to prevent organ rejection and also depends on the availability of a kidney match (Mayo Clinic, 2018). The second option is dialysis, a process involving the artificial filtration of a patient's blood with either a hemodialysis machine or a peritoneal dialysis catheter inserted into the abdomen (Mayo Clinic, 2018). Should a patient choose to discontinue dialysis, the life expectancy of that patient is generally a few months (Mayo Clinic, 2018).

In the case of cadmium toxicity, oxidative damage in the kidneys occurs by means of

metallothionein, the bloodstream protein designed to bind metal ions (Fujiwara *et al.*, 2012). After it binds to cadmium, the cadmium-metallothionein complex travels through the bloodstream and the renal corpuscle into the proximal convoluted tubule, where it is reabsorbed into the first two-thirds of the tubule known as the S1 and S2 segments (Renugadevi and Prabu, 2009). The protein complex is then degraded by the lysosome, and this degradation releases cadmium into those epithelial cells. An accumulation of cadmium, even in low concentrations, will cause oxidative damage to the glomerulus via the production of reactive oxygen species (Wei-ping *et al.*, 2007; Chen *et al.*, 2016). Additionally, chronic exposure to cadmium stimulates fibrosis due to oxidative stress-induced apoptosis and inflammatory signaling (Chen *et al*., 2016; Matovinović, 2009; Pham-Huy *et al.*, 2008; Grynberg *et al.*, 2017). The buildup of scar tissue that occurs with fibrosis interferes with the kidney's ability to function, and this condition can lead to chronic kidney disease (Chen *et al.*, 2016).

**Quercetin and the Power of Antioxidants**

Antioxidants are chemicals that counteract oxidative stress in biological systems (Pham-Huy *et al.*, 2008). Endogenous antioxidants are produced within the biological system under oxidative stress, and these built-in mechanisms are known as the antioxidant system (Pham-Huy *et al.*, 2008; Li *et al.*, 2017). The antioxidant system consists of seven enzymes: superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione-S-transferase, glutathione peroxidase, glutathione reductase, and reduced glutathione (Li *et al.*, 2017). In normal circumstances, these enzymes are sufficient in reversing oxidative damage and making

free radicals harmless. With increased oxidative stress, such as that which cadmium induces, endogenous antioxidants are not able to protect the affected tissue alone (Dua *et al.*, 2015). In those circumstances, exogenous antioxidants supplied from one's diet can assist in protecting cells from the increase in oxidative stress (Anantachoke *et al.*, 2016).

Exogenous antioxidants such as flavonoids have shown promise in protecting cells from cadmium-induced oxidative damage (Donpugha *et al.*, 2011, Pappas *et al*., 2010). Flavonoids are a class of plant and fungal secondary metabolites classified by a 15-carbon skeleton with two phenyl rings and a heterocyclic ring in the middle (Pappas *et al*., 2010). Known to be strong antioxidants and free radical scavengers, this class of molecules can prevent or delay cardiovascular diseases, inflammation, aging, and Alzheimer's disease, among other chronic conditions (Prabu *et al.*, 2013; Pham-Huy *et al.*, 2008). One of the most common flavonoids, found in most fruits and vegetables as well as tea, coffee, and red wine, is quercetin, whose structure is shown in Figure 5 (Kelly, 2011). Quercetin's nomenclature according to the International Union of Pure and Applied Chemistry (IUPAC) is 3,3',4',5,7-pentahydroxy-2-phenylchromen-4-one (Kelly, 2011). Its several hydroxyl groups are the primary reason why it is a strong antioxidant and free radical scavenger. These antioxidant properties are attributed to its ability to chelate transition metal ions, including cadmium (Prabu *et al.*, 2013). Cells show significant uptake of quercetin, particularly in the mitochondria (Kelly, 2011). Quercetin has other beneficial qualities; its effects can cause a decrease in allergies, cancer, blood pressure, and viral infection (Kelly, 2011). Additionally, it has a rich yellow hue that makes its presence easily

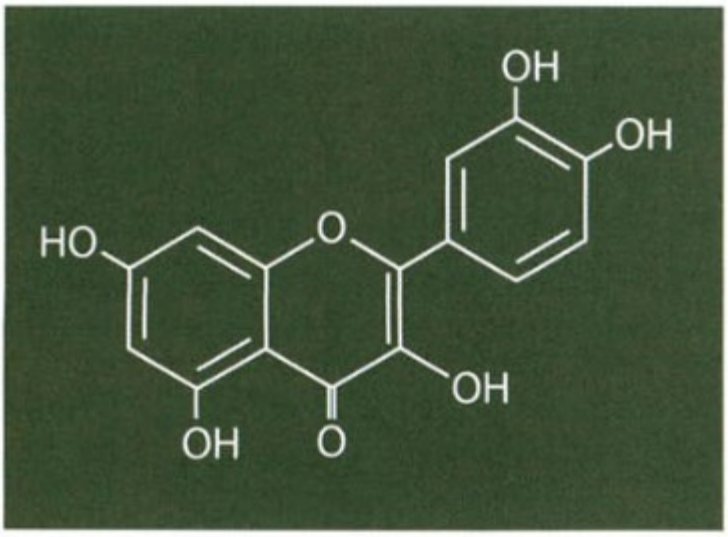


**Fig. 5. Quercetin chemical composition.** (Kelly, 2011)

observable *in vitro*.

Several previous studies *in vivo* have measured protective effects by quercetin in response to cadmium toxicity. For example, Prabu *et al.* demonstrated that quercetin pre-treatment can ameliorate cadmium-induced oxidative pathology in rat cardiovascular systems (2013). In a different study from Dua *et al.*, researchers examined mouse hepatocytes for cadmium-induced oxidative stress after treatment with a plant-aqueous extract rich in quercetin (2015). Their histological analysis showed that the quercetin-rich plant extract pre-treatment effectively kept the tissue almost completely protected from cell death (Dua *et al.*, 2015). Additionally, Li *et al.* reported that a different quercetin-rich plant extract improved bone mineral status in osteoporosis with cadmium exposure (2017).

Compared to these *in vivo* studies, *in vitro* studies can have different conclusions. One such study found that quercetin concentrations of 40 μM and above had cytotoxic effects on ovarian cancer cells *in vitro* (Li *et al.*, 2014). Another *in vitro* study showed that 30 μM quercetin

lowered rat thymocyte growth (Nishimura *et al.*, 2008). Quercetin, therefore, can exhibit antiproliferative activity under certain circumstances (Kelly, 2011). While *in vitro* research is less immediately applicable to human biological systems, it is preferable for elucidating the mechanism of a particular pathway. Because the various conditions surrounding cell growth can be controlled much more closely, researchers who study the JNK pathway have historically used cell lines cultured *in vitro* (Jin *et al.*, 2006; Srivastava *et al.*, 1999; Behrens *et al.*, 1999). The purpose of this thesis, therefore, is fourfold: first, to observe cadmium-induced toxicity in kidney epithelial cells; second, to explore how quercetin affects kidney cell proliferation; third, to determine the effects of quercetin and cadmium in kidney epithelial cells when treated simultaneously; and fourth, to determine if the phosphorylation of JNK is involved in this mechanism for cell death.

## METHODS

### Cell Culture and Solutions for Treatments

HEK 293 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and grown in VWR $T_{25}$ flasks (Radnor, PA) using Eagle's Minimum Essential Medium (EMEM) from Lonza (Miami, FL). Quercetin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Quercetin was diluted using DMSO to make solutions with concentrations of 1 mM, 5 mM, 10 mM, and 20 mM. Cadmium chloride ($CdCl_2$) solutions with the following concentrations were made from stock 10 mM $CdCl_2$ solutions and sterile 10 mM HEPES: 0.2 mM, 0.6 mM, 1 mM, 2 mM, and 4 mM.

### Plating, Treating, and Cell Proliferation Assay

Upon confluence, cells were trypsinized, centrifuged at 2000 xg for 2 min., counted, and seeded in a 96-well plate at 5,000 cells/well with 190 µL of media added to each well (Liu *et al.*, 2015). Cells were allowed to grow for 24 hours, and then treatments were administered. $CdCl_2$ treatments diluted in 10 mM HEPES were added in 10 µL volumes while quercetin concentrations diluted in DMSO were maintained at 2 µL volumes. DMSO and 10 mM HEPES were used as controls. For wells containing both $CdCl_2$ and quercetin, the quercetin pre-treatment was administered 1 hour prior to the $CdCl_2$ treatment (Chen *et al.*, 2016). Each treatment was given in quadruplicate, and treated cells were incubated for 24 hours. Prior to the proliferation assay, the wells were rinsed with media twice to remove all traces of quercetin so that its bright pigment would not interfere with absorption during the proliferation assay. For the

tetrazolium (MTT) cell proliferation assay, the CellTiter 96® Aqueous One Solution from Promega (Madison, WI) was used to measure the rate of respiration following manufacturer's directions. Absorbances were read on a BioTek ELx808 plate reader (Winooski, VT) with Gen5 3.02 software at an absorbance of 490 nm. The MTT assay was run for 5 hours on average.

**Collection of Cell Extracts**

Cells were plated at 600,000 cells/well in 60 $mm^3$ plates and were allowed to grow for 24 hours. Quercetin diluted in DMSO was pre-treated for 1 hour in concentrations of 10 μM, 50 μM, and 100 μM, and $CdCl_2$ treatments diluted in 10 mM HEPES were added to a final concentration of 30 μM. Cell lysates were prepared in RIPA buffer from Sigma Aldrich and with Lipofectin phosphatase and protease inhibitors from Life Technologies (Gaithersburg, MD) according to manufacturer's directions. Cells were washed two times with cold PBS and then lysed. Cell lysates were collected using a cell scraper, sonicated twice for 1 second, and spun at 12,000 rpm for 10 minutes at 4 °C. Supernatant was collected and stored at -80 °C.

**Bradford Assay, Immunoblot Analysis, and Densitometric Analysis**

To measure protein concentrations in cell lysates, bovine serum albumin from Sigma Aldrich was used to prepare a standard curve according to manufacturer's directions. Absorbance of the cell lysates was measured at 595 nm by a Genesys 10 uv spectrophotometer from Fisher Scientific (Hampton, NH). After calculating the protein concentrations, the protein samples (28.9 μg) were separated on a 4-20% polyacrylamide gel at 200 V and transferred to a nitrocellulose membrane.

After blocking with 5% milk in Tris buffer saline with 0.1% Tween 20 for 1 hour, immunoblot analysis was performed using a polyclonal antibody raised to phosphorylated-JNK (Cell Signaling Technology, Danvers, MA) in a 1/1000 dilution overnight. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 hour (Thermo Scientific, Waltham, MA). Bands were visualized using ECL reagents and the Chemidoc Imaging System from BioRad Laboratories (Hercules, CA). The blot was stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and retreated with a polyclonal antibody raised to total-JNK (Cell Signaling Technology) in a 1/500 dilution overnight at 4 °C. Following the secondary antibody, the blot was developed as previously described. Densitometric analysis was performed afterwards with Un-Scan It Graph Digitizer Software (Silk Scientific, Orem, UT) to compare phosphorylated JNK with total JNK, and the results were reported as ratios.

## RESULTS

To determine if quercetin is able to provide protection against cadmium toxicity, it was first important to establish the optimal concentrations of cadmium that induced cell death in human kidney epithelial cells using the cell line HEK 293. Cells were treated with increasing concentrations of cadmium for 24 hours, and a cell proliferation assay was then used to measure the rate of respiration relative to untreated controls. These results are shown in Figure 6.

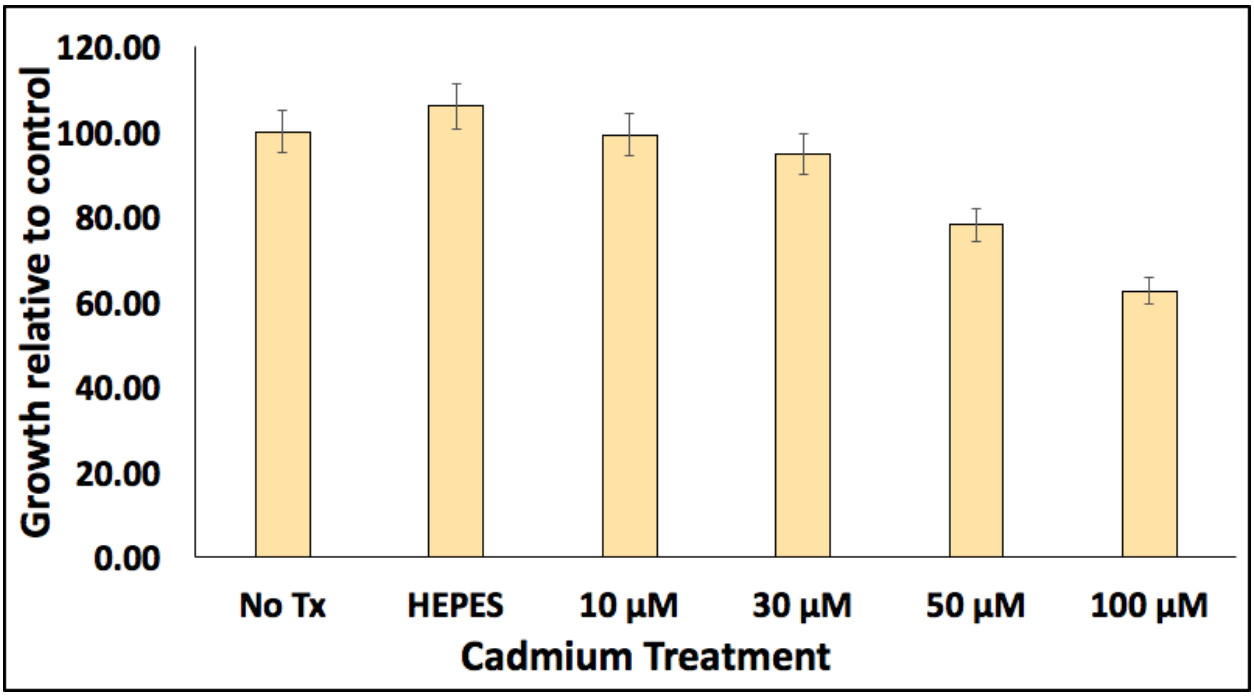


**Fig. 6. Cell proliferation assay with various treatments of cadmium chloride.** Cells were seeded at a cellular density of $5\times10^3$ cells/well. They were then treated with the increasing concentrations of $CdCl_2$ for 24 hours. An MTT assay was performed to measure the rate of cell proliferation (quantified by the absorbance at 490 nm) with each concentration.

Compared to no treatment, 10 mM HEPES (used as the vehicle for cadmium) and 10 µM cadmium chloride have little, if any, effect on cell proliferation. As the concentration of cadmium chloride increases to 30, 50, and 100 µM, however, cell growth was found to decrease,

with the lowest amount of growth coinciding with the 100 µM cadmium chloride treatment. Because 30 µM cadmium initiated an observable toxic response without resulting in immediate cell death, this concentration was used in all of the following experiments.

To determine how quercetin may affect kidney epithelial cell proliferation, HEK 293 cells were seeded and treated with increasing concentrations of quercetin for 24 hours. Cell growth was again measured using a cell proliferation assay. As shown in Figure 7, 10 mM HEPES and DMSO have little effect on cell growth relative to the untreated control.

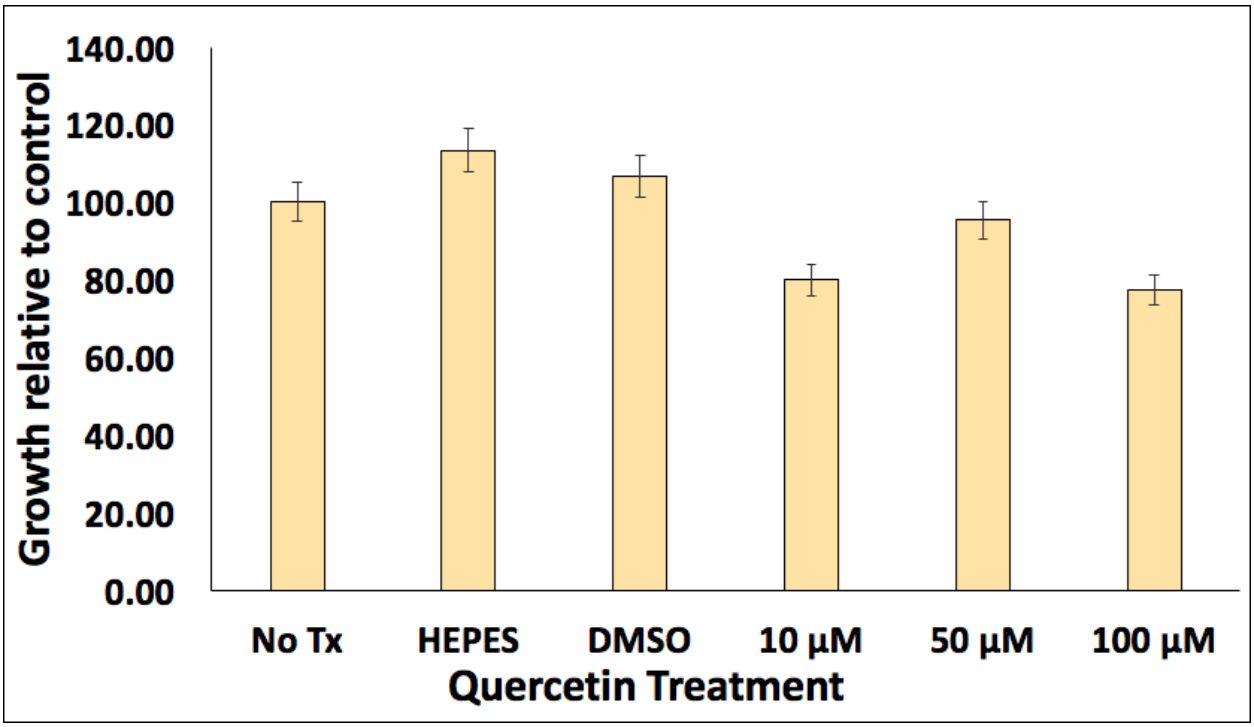


**Fig. 7. Cell proliferation assay with various treatments of quercetin.** Cells were seeded at a cellular density of $5\times10^3$ cells/well 24 hours prior to quercetin treatments. Cells were then treated with the increasing concentrations of quercetin for 24 hours. An MTT assay was performed to measure the rate of cell proliferation (quantified by the absorbance at 490 nm) with each concentration.

Surprisingly, the quercetin treatments decreased cell viability when cells were treated with 10 µM and 100 µM quercetin. The 50 µM quercetin appears to have no effect on growth, but it is

likely that this is related to the methodology used in this study. Quercetin is a yellow pigment, and if not all of the pigment is removed before the proliferation assay, it could skew the absorption readings. Repeated experiments have supported that an increase in quercetin concentration results in increasing cell death (data not shown).

Previous studies have shown that quercetin has a protective effect against cadmium toxicity, yet the results from the present study suggest that quercetin alone decreases cell proliferation. In comparison to cadmium, it is clear that cadmium toxicity has a greater effect on cell growth. To determine if quercetin might protect against the toxic effect of cadmium or synergistically increase cell death, kidney cell proliferation was measured in the presence of both quercetin and cadmium. In this experiment, HEK 293 cells were pre-treated with increasing concentrations of quercetin for 1 hour, followed by treatment with 30 μM cadmium chloride for 24 hours. As shown in in Figure 8, 30 μM cadmium and increasing concentrations of quercetin alone both result in decreased cell growth as previously observed. Treatment with both 30 μM cadmium and increasing concentrations of quercetin shows a slight increase in cell death compared to either alone. This result suggests that there may be a synergistic effect between cadmium and quercetin that further induces cell death.

Other studies have shown that JNK phosphorylation is induced in the presence of cadmium, suggesting that cadmium may induce cell death by activating the apoptotic pathway. To determine if both cadmium and quercetin can activate phosphorylation of JNK, HEK 293 cells were pre-treated with increasing concentrations of quercetin for 1 hour, followed by

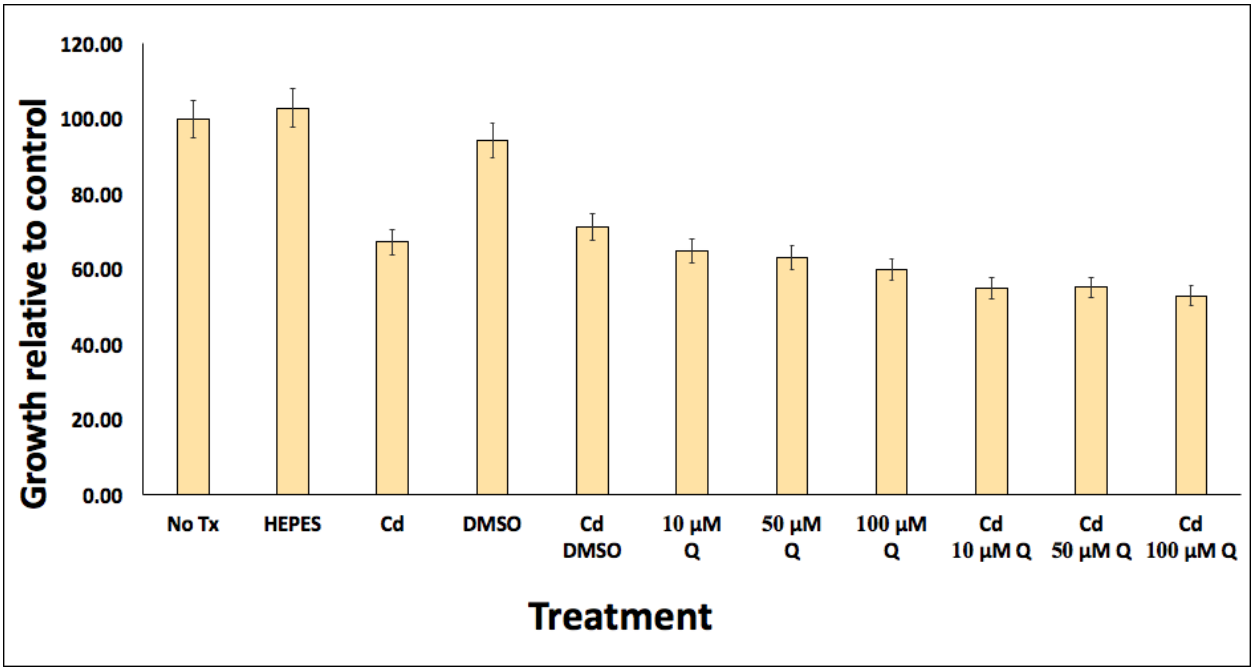


**Fig. 8. Cell proliferation assay with quercetin pre-treatment and cadmium treatment.** Cells were seeded at a cellular density of $5\times10^3$ cells/well 24 hours before quercetin treatments. Cells were treated with quercetin for 1 hour prior to treatment with 30 µM $CdCl_2$. An MTT assay was performed 24 hours later to measure the rate of cell proliferation (quantified by the absorbance at 490 nm) with each treatment.

treatment with 30 µM cadmium chloride for 1 hour. Immunoblot analysis was then performed to measure levels of phosphorylated JNK compared to total JNK as a loading control. As shown in Figure 9, very little JNK is phosphorylated following treatment with 30 µM cadmium. In contrast, JNK phosphorylation is clearly observed following treatment with increasing concentrations of quercetin. When cells were treated with both cadmium and quercetin, the increase in JNK phosphorylation is even greater. (Note that due to a possible mistake in the experimental design, no data was collected from the sample containing cadmium chloride and 50 µM quercetin). To determine the levels of JNK phosphorylation, these results were quantified by densitometry as shown in Figure 10. These results verify that cadmium treatment alone results

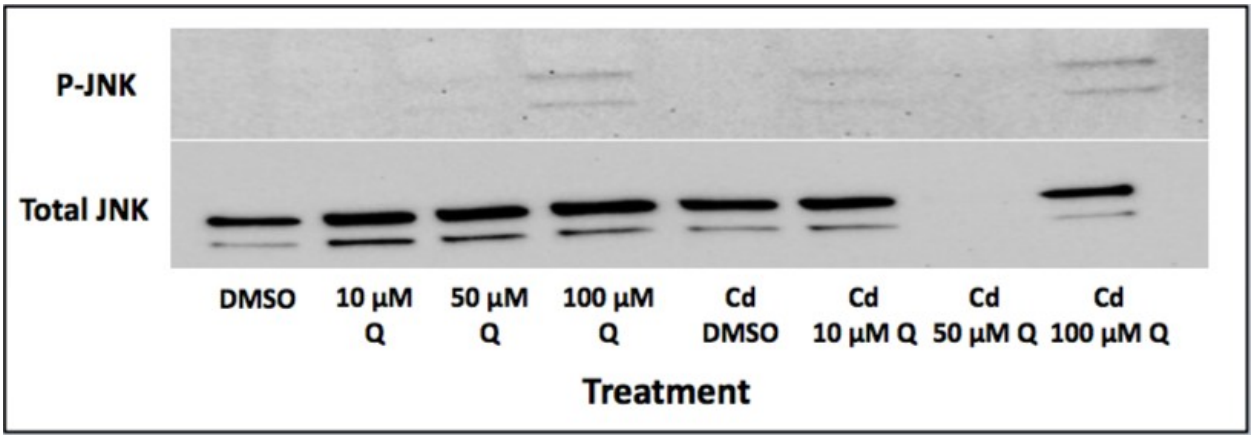


**Fig. 9. Immunoblot analysis of phosphorylated JNK and total JNK in cell lysates.** Cells were plated at $6\times10^5$ cells/well in 60 $mm^3$ plates and were allowed to grow for 24 hours. Treatments were added with the same concentrations in previous experiments.

in a small, 2.3-fold increase in the ratio of P-JNK to total JNK. With increasing concentrations of quercetin, JNK phosphorylation also increases in a concentration-dependent manner. When quercetin pre-treatment is administered in conjunction with cadmium chloride, the P-JNK / total JNK ratio increases even further, reaching a 4.8-fold increase compared to cadmium alone. These results suggest that both cadmium and quercetin activate phosphorylation of JNK and that this activation may be a synergistic effect.

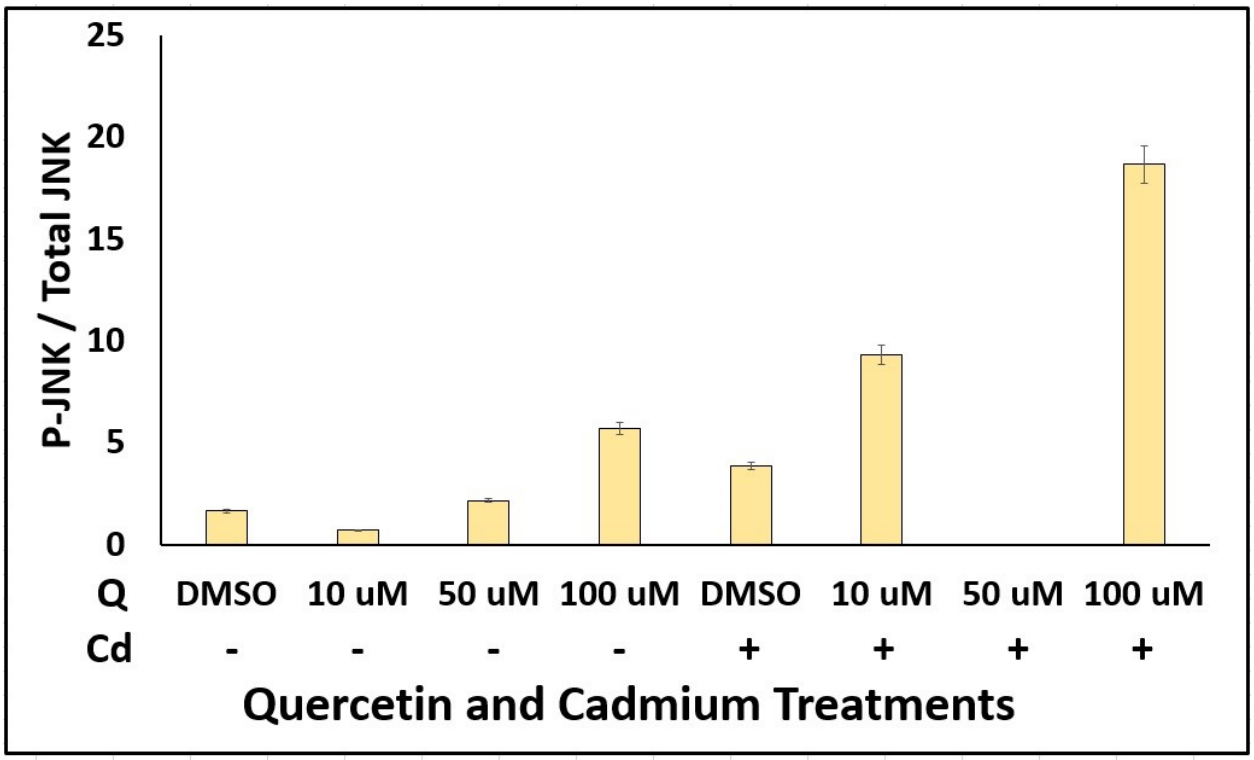


**Fig. 10. Densitometric analysis of phosphorylated JNK and total JNK in cell lysates.** The images of the immunoblot analysis in Figure 9 were analyzed for density using graph digitizer software to calculate the ratios.

## DISCUSSION

To study the effect of cadmium toxicity in HEK 293 cells, it was first important to determine the amount of cadmium chloride necessary to induce toxicity in the HEK 293 human kidney epithelial cell line. After treating cells with 10 - 100 μM cadmium chloride for 24 hours, the results showed that cell proliferation was inhibited in a concentration-dependent manner. This data is consistent with previous published studies addressing cadmium toxicity. Prozialeck *et al.* showed that 15-hour cadmium treatments to human umbilical vein endothelial cells disrupted cell structure in a concentration-dependent manner (2006). In a different study by Wang *et al.*, adult neural progenitor cells from the subventricular zone of rat brain were treated with cadmium for 24 hours, and cell proliferation was shown to decrease due to apoptosis (2017). In the remaining studies in this project, 30 μM cadmium chloride was used because it was strong enough to reduce cell growth but did not completely inhibit viability. This provided an optimal concentration that provided the opportunity to test the effects of quercetin.

In the face of oxidative damage, the use of antioxidants such as quercetin has shown promise in protecting cells from cadmium toxicity. Feeding plant extracts containing quercetin to mice was found to protect the livers of these mice from cadmium toxicity and their bones from osteoporosis (Li *et al.*, 2017; Dua *et al.*, 2015). Also, rats treated with quercetin had healthier cardiovascular systems than rats treated with cadmium (Prabu *et al.*, 2013). Each of these studies, however, were conducted *in vivo*. Previous *in vitro* studies have suggested that quercetin decreases cell viability. Li *et al.* showed that 40 μM and greater concentrations of quercetin and

various chemotherapeutic agents decreased cell viability in an ovarian cancer cell line (2014). Another study by Nishimura *et al.* found that 30 μM quercetin treatments induced apoptosis in rat thymocytes, but their quercetin concentrations of 10 μM and below did not differ significantly from the control treatment (2008). In the present study, it was observed that treatment with 10 - 100 μM quercetin for 24 hours inhibited cellular growth in a concentration-dependent manner.

These previous studies concerning the effects of cadmium and quercetin have conflicting results. At times, quercetin exhibits protective effects in the presence of oxidative damage, but conversely, quercetin itself induces cell death. In the present study, it was found that a one-hour quercetin pre-treatment does not lessen cadmium-induced cytotoxicity. Rather, the combined effect of cadmium chloride and quercetin lowers cell proliferation even further. Based on these observations, it appears that decreased concentrations of quercetin, 10 μM and lower, may protect cells from oxidative damage due to cadmium. Further studies are necessary to determine if concentrations lower than 10 μM have a protective effect. Above 10 μM, however, quercetin compounds the effects of cadmium and increases the levels of cell death.

Regarding cadmium cytotoxicity, previous studies corroborate the involvement of the JNK pathway. Once JNK is phosphorylated, cell death is known to occur, typically by means of apoptosis (Behrens *et al.*, 1999). Wang *et al.* treated rat adult neural progenitor cells with 0.45 μM cadmium chloride and found that the cadmium treatment yielded a twelvefold induction of phosphorylated JNK after 30 minutes (2017). Chen *et al.* also found that treatment of 4 μM cadmium chloride in human renal mesangial cells increased the ratio of phosphorylated JNK to

total JNK from 1 to 10 over a 12-hour period (2016). Additionally, when these human renal mesangial cells were pre-treated with the JNK inhibitor SP600125, cadmium treatment did not significantly change the amount of JNK phosphorylation (Chen *et al.*, 2016).

In this study, it was observed with immunoblot analyses that phosphorylation of JNK increases in the presence of quercetin in a concentration-dependent manner. Phosphorylation of JNK was also observed in the presence of cadmium, but at a much lower effect, with only a two-fold increase in the ratio of phosphorylated JNK to total JNK. At its highest concentration of 100 μM, quercetin alone more strongly induces JNK phosphorylation than cadmium chloride. When the two compounds are both present, the ratio of phosphorylated JNK to total JNK increases even further. These results suggest that both cadmium chloride and quercetin activate the JNK pathway individually at these concentrations, and combining quercetin pre-treatment with cadmium chloride treatment creates a synergistic effect on JNK phosphorylation.

From these studies, it is clear that quercetin induces cell death to the same degree as cadmium chloride, and quercetin pre-treatment prior to cadmium chloride treatment exacerbates the observed cadmium-induced toxicity. Both quercetin and cadmium chloride likely induce cell death through the JNK pathway via JNK phosphorylation. Based on previous reports, phosphorylated JNK then disrupts mitochondrial function and increases the transcription and translation of apoptotic factors. These events in the mitochondria produce reactive oxygen species, which further contribute to the destruction of the cell (Prozialeck *et al.*, 2006).

Apoptosis is a common response if the integrity of the cell is jeopardized, and it is often

necessary to keep the balance of cell division intact (Widmaier, *et al.*, 2014). When this occurs across a section of epithelial tissue in the proximal convoluted tubule, however, that section of nephron becomes dysfunctional. Secretion and reabsorption of the filtrate cannot occur, and the epithelial tissue cannot regenerate without healthy cells present. Renal function decreases overall in this manner, and if an individual is exposed to enough cadmium pollution or contamination over their lifetime, then that individual is condemned to dialysis treatments at the end of their life unless a kidney transplant is a viable option. Understanding the JNK pathway and its interactions with cadmium and quercetin is critical to finding a treatment for kidney failure and for discovering how to take preventative measures for individuals susceptible to cadmium toxicity.

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